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Effectiveness of cell therapy of acute radiation syndrome in mice with intravenous and intraperitoneal administration of a cellular product

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ABSTRACT

The therapeutic effect of fibroblast-like cells obtained from the stromal vascular fraction of subcutaneous adipose tissue of mice and cultured for the treatment of bone marrow form of acute radiation syndrome was studied on a mouse experimental model. The cells were identified as multipotent mesenchymal stem (stromal) cells, owing to adhesion to plastic, confluent monolayer formation during cultivation, and the fact that osteogenic differentiation in vitro resulted in osteoblast maturation and calcium deposit formation, which indicated their multipotent nature. Irradiation of laboratory rodents was conducted using the *X*-ray therapy unit "RUM-17". Stromal cells were obtained from subcutaneous adipose tissue of a mouse and grown in a culture of 3–4 passages and used as a cell product. Cell transplantation was performed 24 h after uniform *X*-ray irradiation of mice at a dose of 7.8 Gy. This is the first study to compare the therapeutic efficacy of allogeneic transplantation of multipotent mesenchymal stem cells with the different routes (intravenous and intraperitoneal) of cell suspension administration. A significant increase was found in the survival rate of mice during the 30-day follow-up period after lethal dose irradiation, which depended on the number of injected cells and delivery method of the biomedical cell product. Thus, with intravenous administration of 30 and 60 × 10^3 multipotent mesenchymal stem cells, the 30-day survival rate of mice after irradiation at a dose of 7.8 Gy increased by 54.5% and 40%, respectively, compared with that of untreated animals ($p = 0.03$). An increase in the number of cells in the cell product to 120 × 10^3/mouse led to a decrease in therapy effectiveness. In intraperitoneal administration, the protection of animals from death was 57% after transplantation of 30 and 60 × 10^3 cells ($p = 0.039$) and 50% after application of 120 × 10^3 cells. On day 30 after irradiation, in the introduction of a cellular product in different schemes, 70%–80% of animals showed restoration of the values of the main indicators of the hematopoiesis system to initial levels. Thus, cell therapy using multipotent mesenchymal stem cells isolated from adipose tissue with intravenous and intraperitoneal delivery routes of the cellular product to the irradiated body protects mice from death after exposure to *X*-ray radiation in lethal doses, decreasing the severity of radiation damage to the hematopoietic system in mice, and provides prospects for further research as an effective and safe treatment for acute radiation sickness.

Keywords: survival; adipose tissue; cell therapy; acute radiation syndrome; *X*-ray radiation; acute radiation sickness; multipotent mesenchymal stem cells; hematopoiesis.

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Эффективность клеточной терапии острого лучевого синдрома у мышей при внутривенном и внутрибрюшинном введении клеточного продукта

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АННОТАЦИЯ

На мышиной экспериментальной модели исследовано терапевтическое действие популяции фибробластоподобных клеток, полученных из стромально-васкулярной фракции подкожной жировой ткани мышей и выращенных в культуре, для лечения костномозговой формы острого радиационного синдрома. Клетки были идентифицированы как мультипотентные мезенхимальные стволовые (стромальные) клетки, поскольку обладали адгезивностью к пластику, формируя при культивировании конфлюэнтный монослой, а процесс их остеогенной дифференцировки *in vitro* завершался созреванием остеобластов и формированием кальциевых депозитов, что свидетельствовало об их мультипотентной природе. Облучение лабораторных грызунов осуществляли с помощью рентгенотерапевтической установки «РУМ-17». В качестве клеточного продукта использовали популяцию стромальных клеток, полученных из подкожной жировой ткани мыши и выращенных в культуре 3–4-го пассажа. Трансплантацию клеток осуществляли через 24 ч после общего относительно равномерного рентгеновского облучения мышей в дозе 7,8 Гр. Впервые сравнивается терапевтическая эффективность аллогенной трансплантации мультипотентных мезенхимальных стволовых клеток при разных путях введения клеточной суспензии — внутривенном и внутрибрюшинном. Показано существенное повышение выживаемости мышей в течение 30-суточного периода наблюдения после облучения в летальной дозе, которое зависело от количества введенных клеток и способа доставки биомедицинского клеточного продукта. Так, при внутривенном введении 30 и 60 × 10^3 мультипотентных мезенхимальных стволовых клеток 30-суточная выживаемость мышей после облучения в дозе 7,8 Гр по сравнению с группой животных без лечения повышалась на 54,5 и 40 % соответственно (p = 0,03). Увеличение численности клеток в клеточном продукте до 120 × 10^3/мышь приводило к снижению эффективности терапии. При внутрибрюшинном введении защита животных от гибели составила 57 % после трансплантации 30 и 60 × 10^3 клеток (p = 0,039) и 50 % после применения 120 × 10^3 клеток. На 30-е сутки после облучения на фоне введения клеточного продукта в разных схемах у 70–80 % животных было отмечено восстановление значений основных показателей системы кроветворения до исходного уровня. Таким образом, клеточная терапия с использованием мультипотентных мезенхимальных стволовых клеток, выделенных из жировой ткани, при внутривенном и внутрибрюшинном путях доставки клеточного продукта в облученный организм обеспечивает защиту мышей от гибели после воздействия рентгеновского излучения в летальных дозах, способствуя снижению тяжести лучевого поражения гемопоэтической системы у мышей, и имеет несомненные перспективы для дальнейших исследований в качестве эффективного и безопасного средства лечения острой лучевой болезни.

Ключевые слова: выживаемость; жировая ткань; клеточная терапия; острый радиационный синдром; рентгеновское излучение; острая лучевая болезнь; мультипотентные мезенхимальные стволовые клетки; гемопоэз.

Как цитировать

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通过静脉注射和腹腔注射细胞产品对小鼠急性放射综合征进行细胞治疗的疗效

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摘要

在小鼠实验模型中，研究了从小鼠皮下脂肪组织的基质血管部分提取并培养的成纤维细胞对急性放射综合征髓质型的治疗效果。这些细胞被鉴定为多能间质干（基质）细胞，因为它们具有与塑料的粘附性，培养时形成汇合单层。细胞在体外的成骨分化过程是通过成骨细胞的成熟和钙沉积的形成来完成的，这表明了细胞的多能性。使用 RUM-17 X 射线治疗装置对实验鼠进行照射。从小鼠皮下脂肪组织中提取并培养到第 3-4 期的基质细胞群被用作细胞产品。在小鼠接受剂量为 7.8 Gy 的相对均匀的 X 射线照射 24 小时后进行细胞移植。首次比较了通过静脉注射和腹腔注射细胞悬液进行多能间充质干细胞异体移植的疗效。结果表明，小鼠在接受致死剂量照射后的 30 天观察期内存活率明显提高，这取决于注射细胞的数量和生物医学细胞产品的给药方法。因此，静脉注射30×103和60×103多能间充质干细胞可提高小鼠在7.8Gy剂量照射后30天的存活率，与未接受治疗的动物组相比，存活率分别提高了54.5%和40%（p = 0.03）。将细胞产品中的细胞数量增加到 120×103 个/只小鼠，会导致疗效下降。腹腔注射时，移植 30×103 和 60×103 细胞后，保护动物免于死亡的比例分别为 57%（p = 0.039），移植 120×103 细胞后，保护动物免于死亡的比例为 50%。在按不同方案注射细胞产品的背景下，在照射后第30天，70%-80%的动物观察到造血系统的基本参数恢复到初始水平。因此，利用从脂肪组织中分离出来的多能间充质干细胞，通过静脉注射和腹腔注射途径将细胞产品输送到受照射的机体中，对小鼠进行细胞疗法，可以保护小鼠在暴露于致命剂量的X射线后免于死亡。这有助于减轻辐射对小鼠造血系统损伤的严重程度，作为一种有效、安全的急性放射病治疗方法，具有不容置疑的进一步研究前景。

关键词：存活率；脂肪组织；细胞疗法；急性辐射综合征；X 射线辐射；急性放射病；多能间充质干细胞；造血。

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BACKGROUND

One of the most promising fields of cell therapy for radiation injuries is using mesenchymal stem cells; however, the available data on their use are limited. Over the past decades, cell therapy for various pathological conditions has been considered an advanced and promising method. This method aims to replace damaged cells and tissue structures in the body, restoring functions in various organs. It is being actively studied by a wide range of medical specialists [1, 2].

One of the most important and relevant fields of application of biomedical cell products is the treatment of acute radiation syndrome (ARS) [3, 4], a severe life-threatening condition that develops after irradiation of the entire body (or most of it) for a relatively short period. The most serious complications after acute radiation exposure are associated with damage to the bone marrow and hematopoietic system, which occurs after irradiation at doses of 4–6 Gy and can become fatal to the body due to the high probability of infectious complications and hemorrhagic syndrome [5–7].

A generally accepted pathogenetically substantiated method of anti-radiation cell therapy is bone marrow or allogeneic hematopoietic stem cell transplants. However, the first clinical trials have shown that they can induce severe complications, often leading to lethal outcomes, and are not effective enough under conditions of complete myelosuppression [3, 7].

These failures have prompted researchers to search for new promising strategies, particularly the use of multipotent mesenchymal stem (stromal) cells (MMSC). The main physiological function of MMSCs is maintaining a protective and regenerative microenvironment for hematopoietic stem cells. Clinical interest in MMSCs has increased significantly after discovering their immunoprivileged properties, allowing them to be used in allogeneic transplantations. The potential success of using MMSCs for the treatment of ARS is attributed to their low immunogenicity, secretory activity, and ability to produce a wide range of cytokines and growth factors necessary for the proliferation and differentiation of hematopoietic precursors [3, 8–11].

The main source of MMSCs has long been bone marrow, but in recent years, most scientific research focused on studying the therapeutic efficiency of MMSCs isolated from more accessible sources, namely, placenta and placental blood, umbilical cord connective tissue, and adipose tissue [3].

Currently, the biomedical cell product PLX-R18 from Pluri Inc. (Israel) is being developed in collaboration with the Ames Research Center (USA), which is a culture of human placental cells with a mesenchymal cell phenotype for the treatment of ARS, as well as graft-versus-host disease and hematopoietic cell engraftment syndrome [4]. In the Russian Federation, there are no registered medicinal products created based on cellular technologies, and work in the field of cellular therapy of radiation injuries is extremely limited; therefore, research aimed at the possibility of obtaining an optimal cellular product for the treatment of ARS is of undoubted relevance and scientific and practical significance.

The study aimed to evaluate experimentally the efficiency of cell therapy for the bone marrow form of ARS in mice using different routes of administration of a population of stromal cells obtained from adipose tissue.

MATERIALS AND METHODS

The study was conducted *in vivo* using a model of the bone marrow form of ARS in laboratory mice. Allogeneic stromal cells obtained from adipose tissue were used as a cellular product. Cell therapy was performed 24 h after the general irradiation of mice at a lethal dose (LD). Group formation for the study was performed through block randomization using a computer random number generator. Experimental groups included animals exposed to *X*-ray radiation with different types of cell therapy; the "control–irradiation" group (C irr) consisted of animals irradiated without treatment, and the group (C biol) consisted of intact mice. Surviving mice were sacrificed after a 30-day follow-up period, necropsy was performed, and blood samples were collected for hematologic studies. The efficacy of cell therapy was assessed by comparing survival parameters and some morphofunctional characteristics in animals during its implementation with control groups.

The experiments were performed using 120 white outbred male mice weighing 20–25 g, obtained from the laboratory animal nursery "Rappolovo" of the National Research Center "Kurchatov Institute" (Leningrad region). The animals were kept under standard vivarium conditions, with 12 animals per cage, and had free access to water and food. The light regime was 12 × 12 h. Feeding and caring for the animals were performed before midday, with complete feed used for feeding. After being received from the nursery, the animals underwent a 14-day quarantine; individuals showing signs of disease or other damage were excluded from the study.

Manipulations for obtaining tissue from laboratory rodents isolating cells and working with cell culture were performed under aseptic conditions in a culture laboratory with a vertical airflow in a laminar flow bench from Faster (Italy). Subcutaneous adipose tissue isolated from the abdominal region of a laboratory mouse was placed in Petri dishes, washed with 0.9% sodium chloride solution, and thoroughly

crushed using sterile instruments. Decomposition of adipose tissue fragments was performed using a 0.1% solution of collagenase from crab hepatopancreas in Dulbecco's buffer without Ca and Mg, produced by BioloT (Russia), under constant stirring on a magnetic stirrer for 40 min at 37°C. At the end of the process, to inactivate the enzyme, Dulbecco's modified Eagle's medium (DMEM) nutrient medium from Gibco (USA) with 10% fetal bovine serum (FBS) from Gibco (USA) was added to the resulting suspension, which was then centrifuged at 400 g for 10 min. The resulting cell sediment was washed three times in Hanks' solution produced by BioloT (Russia) by centrifugation at 400 g for five min. Washed stromal cells were resuspended in DMEM medium with the addition of 10% FBS, 50 μg/mL gentamicin produced by BioloT (Russia), 300 μg/mL L-glutamine produced by BioloT (Russia), and then transferred into Jet Biofil culture flasks (China) and cultivated at 37°C in an atmosphere of 5% carbon dioxide (CO_2) and 80% humidity in a CO_2 incubator from Shellab (USA). After 48–72 h, the cell culture was carefully washed to remove unattached cells using Hanks' medium and then filled with fresh nutrient medium. When 70%–90% confluence was reached, the cells were removed from the plastic carrier surface using a Trypsin–Versene solution produced by BioloT (Russia) in a 1:1 ratio, resuspended in the DMEM nutrient medium (1:3), and distributed into new carriers with an inoculation density 3–5 × 10^4 cells/cm^2.

Cell viability was determined by staining cells with a 0.4% trypan blue solution and counting live and dead cells in a Goryaev chamber. Microscopy and photographing of objects were performed using an AxioVert.A1 FL LED inverted microscope, an Axiocam 503 color digital camera, and Zen 2 (Blue) software from Carl Zeiss (Germany).

The source of ionizing radiation for irradiating experimental animals was the RUM-17 *X*-ray therapy unit produced by Mosrentgen (Union of Soviet Socialist Republics). For irradiation, mice were placed in separate cells of plastic containers mounted on a rotating stand (kymograph). The absorbed dose was 7.8 Gy, corresponding to an LD of 70–90/30 as the dose of ionizing radiation causing death as a result of the bone marrow form of ARS in 70%–90% of irradiated animals during a 30-day follow-up period. Irradiation was performed in the "back-chest" direction with a skin-focal distance of 50 cm, at a voltage of 180 kW, a current of 14 mA, with a filter of 0.5 mm Cu + 1 mm Al. The radiation dose rate was 0.328 Gy/min. Animals from different groups were simultaneously irradiated. Animals in the biological control group were subjected to "false" irradiation, where they were placed in plastic containers on a kymograph and kept under the deactivated anode tube of the installation for the same duration as the experimental groups.

For 30 days after radiation exposure, the general condition of irradiated animals was monitored daily, and the dead animals were recorded; body weight was measured twice a week.

Cell culture at passage levels 3–4 was administered to animals. The monolayer was removed from the surface of the plastic carrier using a Trypsin–Versene solution, resuspended in an isotonic sodium chloride solution, and washed three times by centrifugation at 400 g for 10 min. The number of cells required for administration to animals was obtained by counting in a Goryaev chamber and diluting the suspension to a given concentration with a 0.9% sodium chloride solution. Cell transplantation was performed 24 h after irradiation in an amount of 30, 60, or 120 × 10^3/animal in a 0.3 mL/mouse volume by injection into the tail vein or the abdominal cavity.

On day 30 after irradiation, the surviving animals were euthanized with preliminary premedication by intramuscular administration of Zoletil 100 from Virbac (France) at a dose of 2 mg/kg. Peripheral blood samples of 40 μL were collected into vacutainer tubes with the anticoagulant ethylenediaminetetraacetic acid.

The efficacy of cell therapy was assessed by comparing the parameters of the 30-day survival of irradiated mice, namely, the dynamics of death, the proportion (%) of surviving animals in groups, and the average life expectancy of dead mice. Hematologic studies were conducted using a MicroCC-20Plus-Vet veterinary automatic hematologic analyzer made by High Technology (USA), which enables the quantitative determination of erythrocytes, platelets, and leukocytes counts, the ratio of their main populations, and the levels of hemoglobin and hematocrit. In addition, a complete necropsy of the experimental animals was performed. The internal organs were weighed to determine their mass index, which was calculated as the ratio of the organ mass to the animal's body weight, expressed as a percentage of the total body weight.

A statistical analysis of the obtained data was performed using the Statistica 8.0 application package. The survival of irradiated mice was assessed using the Kaplan – Meier method. To compare the 30-day survival and life expectancy of mice that died from irradiation in the experimental and control groups, the Gehan – Wilcoxon and Fisher's exact tests (two-tailed test) were used. Kruskal – Wallis test or χ^2 (for multiple comparisons), Wilcoxon test for intergroup comparisons, and Mann – Whitney *U* test for assessing intragroup differences were also employed. The results are presented as $M \pm \sigma$, where M is the average value of the indicator, σ is the standard deviation, or as Me [Q_{25}; Q_{75}], where Me is the median, Q_{25} is the lower quartile, and Q_{75} is

the upper quartile. Differences were considered statistically significant at $p < 0.05$.

All manipulations with experimental animals were performed in compliance with the rules for the humane treatment of laboratory animals, as defined by Directive 2010/63/EU of the European Parliament and the Council of the European Union for Animal Welfare [11]. The study was part of the research work "Experimental substantiation of the prospects of using cell therapy in the treatment of the bone marrow form of acute radiation syndrome".

RESULTS AND DISCUSSION

Characteristics of a population of cells obtained from adipose tissue of laboratory mice. Before starting *in vivo* experiments, a population of cells obtained from the subcutaneous adipose tissue of laboratory mice was identified. It is known that as a result of treating adipose tissue with collagenase, a stromal-vascular fraction is obtained, and approximately 2% of the cellular composition of which is stem cells. The main property of stem cells is their ability to differentiate into different cell lines, i.e., "potency" [12]. The Committee of the International Society for Cellular Therapy introduced a system for identifying (MMSC), including the source of origin of MMSC (bone marrow, adipose tissue, etc.) and the minimum criteria for their identification [13]:

1) adhesiveness to plastic when cultivated under standard conditions;

2) expression of specific surface antigens, namely adhesion molecules CD105, CD73, and CD90; lack of expression of CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR;

3) the ability to differentiate *in vitro* into osteoblasts, adipocytes, and chondroblasts.

The cells obtained from the adipose tissue of laboratory rodents had a fibroblast-like shape and were fully adhesive to plastic; a confluent monolayer was formed on the plastic surfaces of culture flasks and plates during cultivation (Fig. 1).

To confirm the ability of the obtained cells for osteogenic differentiation, the cell suspension was exposed to a differentiation medium, which was prepared based on the DMEM nutrient medium from Gibco (USA) with 10% FBS from Gibco (USA), 50 μg/mL gentamicin from BioloT (Russia) and 2 mM L-glutamine produced by BioloT (Russia) by adding dexamethasone from KRKA (Slovenia) at a concentration of 0.04 mg/L, β-glycerol phosphate from SIGMA (Sweden) at a concentration of 2.16 g/L and ascorbic-L(+) acid produced by DIAEM (Russia) at a concentration of 0.05 g/L.

The cell culture at passage level 4 was inoculated into 96-well plates from Orange Scientific (Belgium) at a concentration of 3×10^4 cells/100 μL; 18–24 h after the formation of a monolayer in the experimental wells, the medium was changed to a differentiation medium two times a week. Wells, where cells were cultured in a normal nutrient medium without the addition of factors stimulating osteogenic differentiation, were used as control wells. During cell cultivation for 21 days, the manifestation of visual signs of modification of cells into osteoblasts under the influence of differentiation stimulators was recorded using object microscopy (Fig. 2).

After cultivation, mineralization was assessed by staining cells with alizarin red and the von Kossa method.

Alizarin red staining. After removing the culture medium, 50 μL of a 4% paraformaldehyde solution was added to each well with a monolayer of cells and incubated for 30 min at 37°C. The wells were then washed three times with distilled water from the fixative. Next, 50 μL of a 2% alizarin red

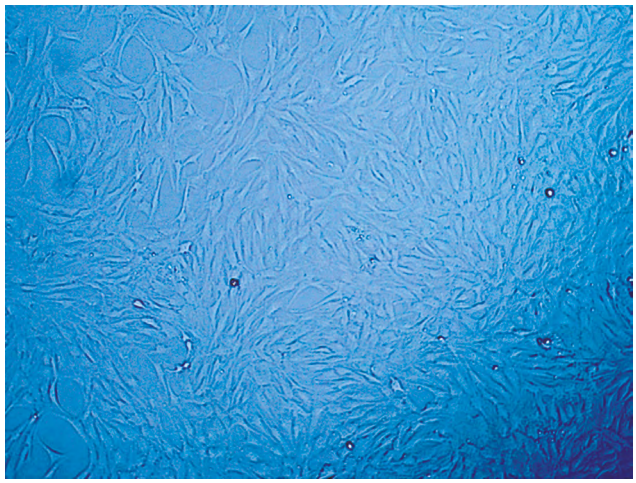
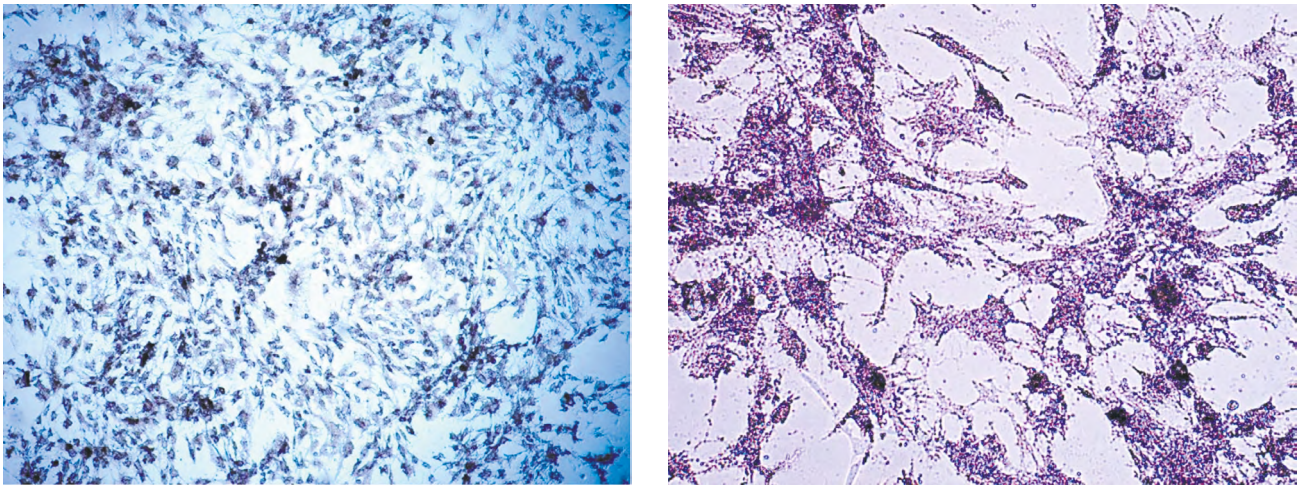


Fig. 1. Monolayer of stromal cells obtained from the adipose tissue of a laboratory mouse, incr. × 10
Рис. 1. Монослой стромальных клеток, полученных из жировой ткани лабораторной мыши, ув. × 10

solution was added to each well. After 5 min of exposure, the stain was thoroughly washed, and 50 μL of acetone was added for 20 s. Microscopy of objects revealed red-stained inorganic calcium salts, indicating osteogenic differentiation of cells (Fig. 3, *a, c*).

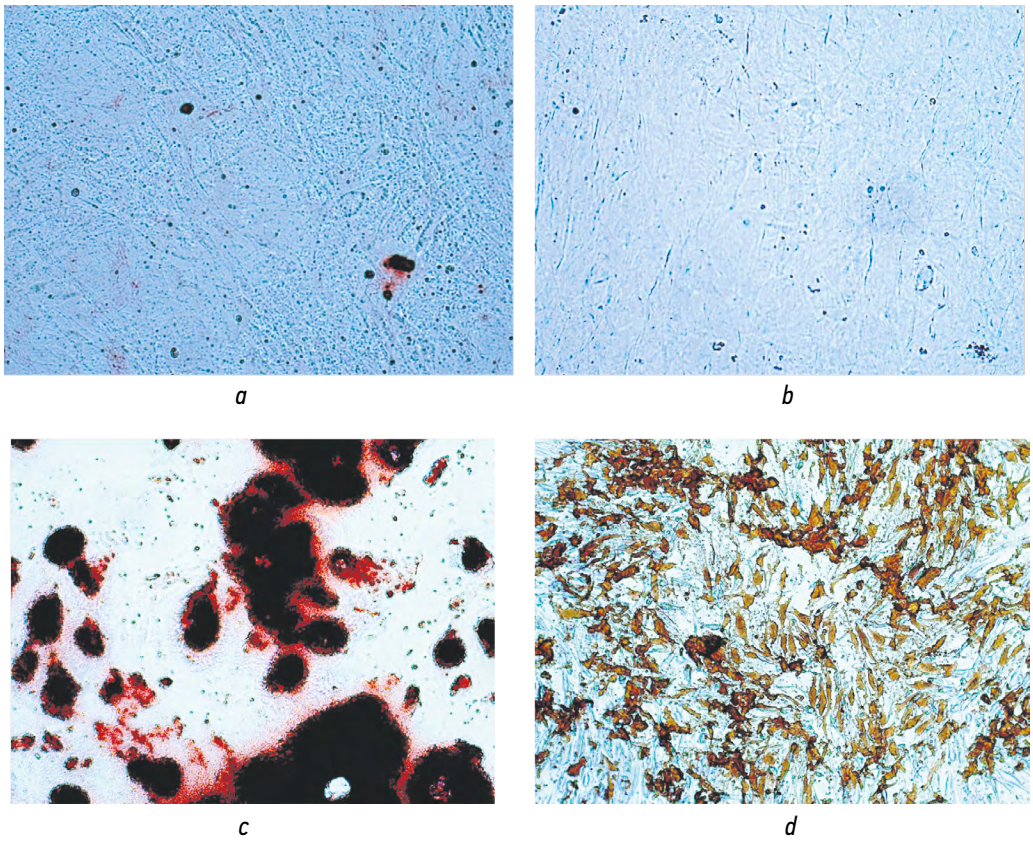
Von Kossa staining. The culture medium was removed from the plate wells with cells, and the cells were fixed with 4% paraformaldehyde for 30 min. A 5% aqueous solution of silver nitrate was added to the wells, which had been washed with distilled water, and the plate was exposed to



a *b*

Fig. 2. Modification of stromal cells obtained from mouse adipose tissue during osteogenic differentiation into osteoblasts: *a* — incr. × 5; *b* — incr. × 20

Рис. 2. Модификация стромальных клеток, полученных из жировой ткани мыши, в процессе остеогенной дифференцировки в остеобласты: *a* — ув. × 5; *b* — ув. × 20



a *b*

c *d*

Fig. 3. Calcium deposits in osteogenic differentiation of stromal cells obtained from mouse adipose tissue: *a*, *b* — control; *c*, *d* — cultivation in a differentiation medium, incr. × 10. Staining: *a, c* — alizarin red; *b, d* — by the von Koss method

Рис. 3. Депозиты кальция в процессе остеогенной дифференцировки стромальных клеток, полученных из жировой ткани мыши: *a*, *b* — контроль; *c*, *d* — культивирование в дифференцировочной среде, ув. × 10. Окрашивание: *a*, *c* — ализариновым красным; *b*, *d* — по методу фон Косса

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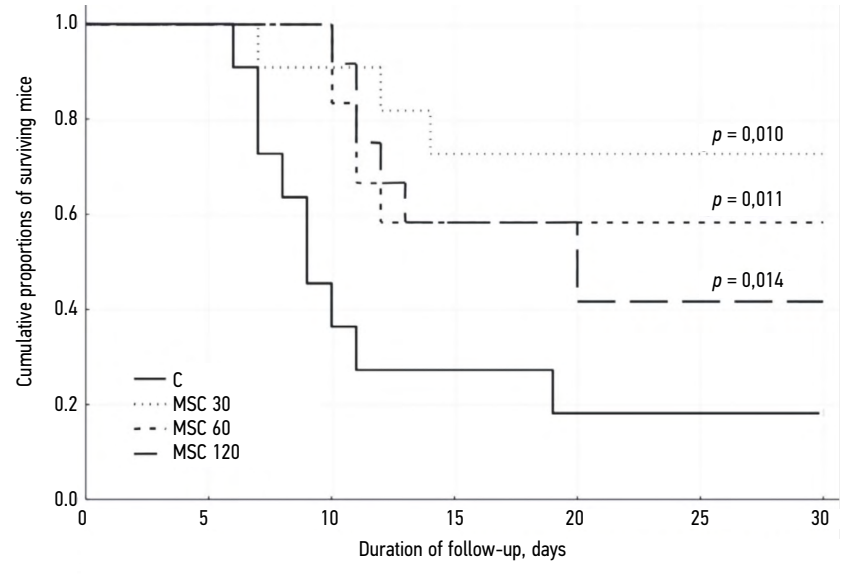
sunlight for 30 min. After thoroughly washing the reagent with distilled water, the cells were exposed to a 5% aqueous solution of sodium thiosulfate for 5 min. The wells were then washed again and examined under a microscope to record the staining results. The mineral deposits were dark brown (Fig. 3, *b, d*).

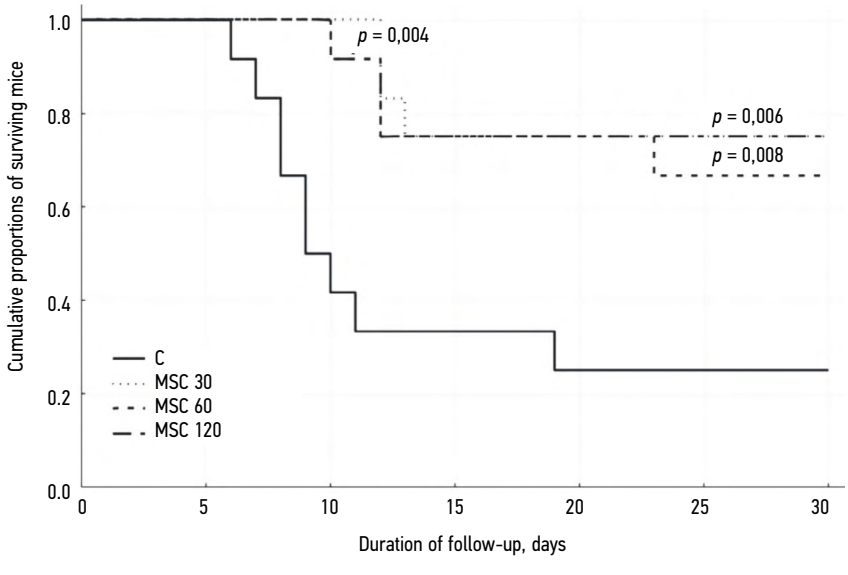
The data obtained after two types of tests demonstrated the presence of calcium deposits in all experimental wells of the plate and their absence in the control ones (Fig. 4).

Thus, the osteogenic differentiation of cells obtained from adipose tissue, characterized by fibroblast-like morphology and adhesiveness to plastic when cultivated under standard conditions, resulted in the maturation of osteoblasts and the formation of calcium deposits. Although the expression of surface antigens specific to MMSCs was not confirmed due to the lack of immunophenotyping possibility, based on the data obtained, cells isolated from the stromal-vascular fraction of laboratory mice were further designated as MMSCs from adipose tissue.

A study of the efficiency of MMSCs obtained from adipose tissue in acute radiation injury was performed in two experiments. In experiment 1, MMSCs from adipose



a



b

Fig. 4. Survival curves of mice after total irradiation at a dose of 7.8 Gy without treatment (K) or with administration 24 h after irradiation of stromal cells isolated from mouse adipose tissue (MSC) in the amount of 30, 60, and 120 thousand cells/mouse: *a* — intravenous; *b* — intraperitoneal

Рис. 4. Кривые выживаемости мышей после общего облучения в дозе 7,8 Гр без лечения (К) или с введением через 24 ч после облучения стромальных клеток, выделенных из жировой ткани мыши (МСК), в количестве 30, 60 или 120 тыс. клеток/мышь: *a* — внутривенно; *b* — внутрибрюшинно

tissue were injected into mice in different quantities (30,000, 60,000, or 120,000 cells/animal, corresponding to 1.1 ± 0.07, 2.2 ± 0.15, and 4.3 ± 0.12 × 10^6 cells/kg, respectively) into the tail vein. In experiment 2, the intraperitoneal route of delivery of the cell suspension in the same doses was used. MMSCs were administered in a 0.9% sodium chloride solution 24 h after irradiation at a dose of 7.8 Gy. At the same time, a physiological solution in a volume of 0.3 mL/mouse was injected into animals in control groups using the appropriate method.

The 30-day survival curves of mice after irradiation at a dose of 7.8 Gy are presented in Fig. 4. These curves indicate that the use of MMSCs had a therapeutic effect in both cases, contributing to a significant decrease in the death of irradiated animals in comparison with control groups. Intergroup differences, assessed using the χ^2 criterion, were statistically significant for both methods of administering the cell suspension ($p = 0.012$ after intravenous administration and $p = 0.004$ after intraperitoneal injection). Moreover, in the case of intravenous administration of the cell product, with an increase in the counts of MMSCs, a decrease in the therapeutic effect was noted. The dose-effect relationship was inversely proportional, but differences between the groups when using different amounts of MMSC were not statistically significant. Administration of MMSCs of 30,000 cells/mouse increased 30-day survival by 53.5% compared with the group of mice without treatment; therapy using 60,000 cells/mouse increased it by 40%; and transplantation of 120,000 cells/mouse contributed to an increase in survival rate by 23.5%. With intraperitoneal administration of MMSCs, the counts of cells did not significantly affect the value of this indicator; cell therapy provided protection from death in 67%–75% of animals (Table 1).

The average life expectancy of mice that died after *X*-ray irradiation at a dose of 7.8 Gy without treatment was nine [7; 10] days. Cell therapy induced a delay in the death of rodents from the bone marrow ARS up to 11–12 days. A significant effect of MMSC on this survival parameter in comparison with the control group was detected with intravenous administration of the cell product with counts of 60,000 and 120,000 cells and with counts of 30,000 and 60,000 cells/mouse in case of intraperitoneal administration (Table 1).

It should be noted that during the 30-day follow-up period, the general condition of the animals was monitored daily, and they were weighed twice a week. The dynamics of body weight in mice of the experimental and control groups are presented in Fig. 5.

The data showed that mice in the C biol group experienced a constant increase in body weight, averaging a 5% gain per week; the increase was more than 20% from the initial level by day 30. After exposure to *X*-ray radiation, the opposite dynamics were registered. The maximum loss of body weight in irradiated mice of all groups from 17% to 23% was recorded during the height of acute radiation injury.

With the intravenous administration of MMSCs, by day 30 after lethal irradiation, restoration of body weight to the initial level was noted in a group of mice using the minimum counts of MMSCs in the experiment (30,000 cells/mouse). The change in body weight of mice after irradiation and intraperitoneal administration of MMSCs was similar. On day

Table 1. Parameters of 30-day survival of mice after total irradiation at a dose of 7.8 Gy and administration of stromal cells isolated from mouse adipose tissue (MMSC, 30, 60, and 120 thousand cells/mouse)
Таблица 1. Параметры 30-суточной выживаемости мышей после общего облучения в дозе 7,8 Гр и введения стромальных клеток, выделенных из жировой ткани мыши (ММСК, 30, 60 или 120 тыс. клеток/мышь)

Animal group	Died/survived	Survival rate, %	Life expectancy of dead mice, days, *Me* [Q_{25}; Q_{75}]
Intravenous administration of MMSC			
7.8 Gy + MMSC 30,000 cells/mouse	3/8	72.7 ± 13.4*	12 [7; 14]
7.8 Gy + MMSC 60,000 cells/mouse	5/7	58.3 ± 14.2	11 [10; 11]$^{\#}$
7.8 Gy + MMSC 120,000 cells/mouse	7/5	41.7 ± 14.2	12 [11; 20]$^{\#\#}$
7.8 Gy (C irr)	9/2	18.2 ± 11.6	9 [7; 10]
Intraperitoneal administration of MMSC			
7.8 Gy + MMSC 30,000 cells/mouse	3/9	75 ± 12.5*	12 [12; 13]$^{\#\#\#}$
7.8 Gy + MMSC 60,000 cells/mouse	3/9	75 ± 12.5*	12 [11; 17.5]$^{\#\#\#\#}$
7.8 Gy + MMSC 120,000 cells/mouse	4/8	67 ± 13.6	12 [10; 12]
7.8 Gy (C irr)	9/3	25 ± 12.5	9 [8; 10]

Note: Differences compared to the K group: * — $p = 0,03$ 03 (Fisher's exact test); $^{\#}$ — $p = 0.009$; $^{\#\#}$ — $p = 0.004$; $^{\#\#\#}$ — $p = 0.05$; $^{\#\#\#\#}$ — $p = 0.03$ (Mann – Whitney *U* test).

Примечание: различия по сравнению с группой К обл: * — $p = 0,03$ (точный критерий Фишера); $^{\#}$ — $p = 0,009$; $^{\#\#}$ — $p = 0,004$; $^{\#\#\#}$ — $p = 0,05$; $^{\#\#\#\#}$ — $p = 0,03$ (*U*-критерий Манна – Уитни).

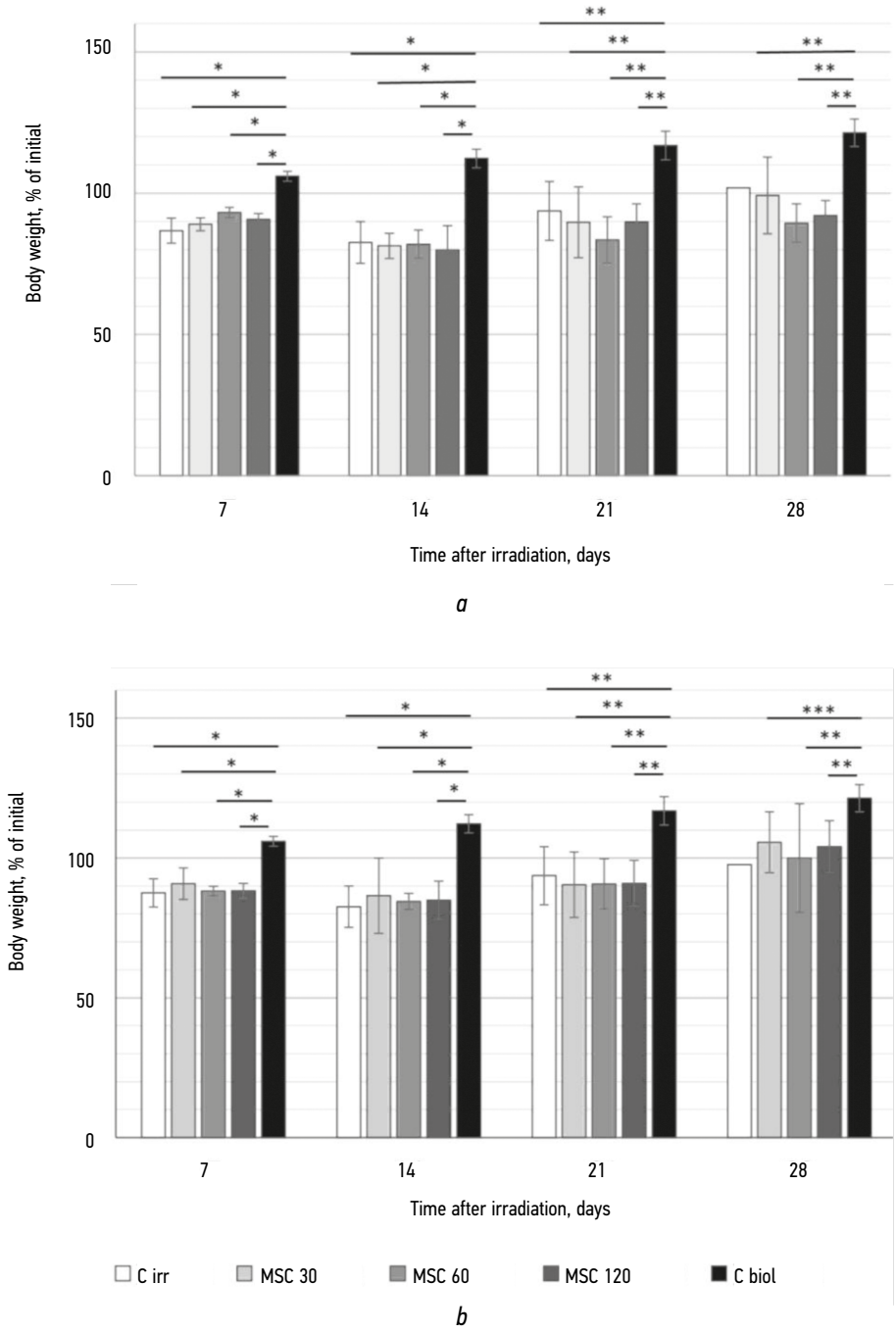


Fig. 5. Body weight change in mice: intact (To biol), after irradiation at a dose of 7.8 Gy without treatment (To vol), or with the introduction of stromal cells isolated from mouse adipose tissue (MSC, 30, 60, and 120 thousand cells/mouse): *a* — intravenous; *b* — intraperitoneal. Differences compared to the K biol group: * — $p < 0.001$; ** — $p < 0.01$; *** — $p < 0.05$ (Mann – Whitney *U* criterion)
Рис. 5. Изменение массы тела у мышей: интактных (К биол), после облучения в дозе 7,8 Гр без лечения (К обл) или с введением стромальных клеток, выделенных из жировой ткани мыши (МСК, 30, 60 и 120 тыс. клеток/мышь): *a* — внутривенно; *b* — внутрибрюшинно. Различия по сравнению с группой К биол: * — $p < 0{,}001$; ** — $p < 0{,}01$; *** — $p < 0{,}05$ (*U*-критерий Манна – Уитни)

30 after irradiation, the initial level of body weight was achieved in 77% of surviving animals that received treatment with MMSCs, regardless of the counts of cells.

Data on the cellular composition of the peripheral blood of animals in the control and experimental groups on day 30 after 7.8 Gy *X*-ray exposure are presented in Table 2. The results indicate that by the end of the follow-up period, the levels of leukocytes and platelets in irradiated animals had not been restored to biological control indicators. However, in more than 50% of irradiated animals, the levels of these blood cells exceeded the lower limit of the physiological norm for rodents of this species. At the same time, intraperitoneal injections of a suspension of MMSCs ensured the restoration of the counts of leukocytes and platelets more effectively

Table 2. Cellular composition of peripheral blood of mice on day 30 after total irradiation at a dose of 7.8 Gy without treatment and with intravenous or intraperitoneal administration of MMSCs isolated from mouse adipose tissue, *Me* [Q_{25}; Q_{75}]
Таблица 2. Клеточный состав периферической крови мышей на 30-е сутки после общего облучения в дозе 7,8 Гр без лечения и с внутривенным или внутрибрюшинным введением ММСК, выделенных из жировой ткани мыши, *Me* [Q_{25}; Q_{75}]

Animal group		Leukocytes, $\times10^9$/L (norm 3.5–9)	Erythrocytes, $\times10^{12}$/L (norm 7–11)	Platelets, $\times10^9$/L (norm 200–800)
Intact		8.8 [8.1; 9.5]	9.1 8.7; 9.3]	400 [353; 452]
Control 7.8 Gy		3.1 [2.4; 3.9]*	6.0 [5.0; 6.9]*	204 [141; 269]*
7.8 Gy + MMSC 30,000 cells/mouse	i.v.	4.2 [4.1; 4.8]*	7.8 [6.8; 8.3]*	172 [165; 182]*
	i.p.	3.6 [3.1; 3.8]*#	8.1 [7.4; 8.3]#	304 [253; 339]
7.8 Gy + MMSC 60,000 cells/mouse	i.v.	3.1 [2.6; 3.4]*	6.1 [5.3; 8.1]	308 [227; 330]*
	i.p.	5.3 [3.9; 5.5]*#	7.6 [7.6; 8.2]#	357 [334; 412]#
7.8 Gy + MMSC. 120,000 cells/mouse	i.v.	2.6 [2.5; 2.9]*	4.6 [4.3; 8.6]*	219 [157; 263]*
	i.p.	4.3 [4.2; 4.9]*#	9.0 [8.4; 9.0]#	317 [277; 377]#

Note: i.v. — intravenous; i.p. — intraperitoneal; * — differences compared to the intact group; # — compared to the control 7.8 Gy group, $p < 0.05$ (Mann – Whitney *U* test).

Примечание: в/в — внутривенно; в/б — внутрибрюшинно; * — различия по сравнению с интактной группой; # — по сравнению с контрольной 7,8 Гр группой, $p < 0,05$ (*U*-критерий Манна – Уитни).

than the intravenous route of delivery of the cellular product, and significant differences compared with the control group were detected after the administration of stromal cells in all doses.

Cell therapy using MMSCs also contributed to a more rapid restoration of the erythrocyte counts in the blood of irradiated animals, particularly with intraperitoneal administration of the cell product. Changes in hemoglobin and hematocrit levels had a similar direction and severity (results not presented). Notably, the lowest data on the assessed parameters were obtained with intravenous administration of MMSC in the amount of 120×10^3 cells.

The persisting leukopenia was mainly due to a decrease in lymphocyte counts. Thus, with intravenous administration of MMSCs in 30,000 cells/mouse and over the entire dose range with intraperitoneal administration of a cell suspension, an increase in their level was noted in comparison with untreated control. The quantitative content of monocytes in mice of all experimental groups differed insignificantly from that of intact animals. The level of neutrophils in the blood of irradiated mice on day 30 after radiation exposure was restored to the level in animals of group C biol, regardless of the route of the cellular product delivery, when MMSC was administered in an amount of 60,000 cells/mouse (Table 3).

Thus, on day 30 after irradiation in animals that received MMSCs, an improvement in peripheral blood parameters was recorded, while the cell product administration into the abdominal cavity contributed to an increase in the efficiency of cell therapy.

Macroscopic assessment of the state of the internal organs of laboratory animals on day 30 after irradiation at a dose of 7.8 Gy and cell therapy with intravenous or intraperitoneal administration of allogeneic MMSCs in different doses showed that the most significant pathological changes occurred in the lungs, while the least significant changes were registered in the spleen. Most irradiated mice, without treatment or after therapy, showed an increase in the weight and volume of the lungs. This was caused by inflammatory processes in the lung tissue, which was reflected in a significant increase in the mass index values of the organ compared with animals in the C biol group (Fig. 6).

The values of the spleen mass index in irradiated mice with different types of cell therapy are illustrated in Fig. 7. In intact animals, the spleen weight was 196 [160; 202] mg, and the splenic mass index was 6.06% [5.67%; 6.75%]. On day 30 after irradiation, the weight and mass index of this organ in animals administered with MMSCs for therapeutic purposes differed significantly depending on the method of delivery of the cellular product. With intravenous administration, the values of these indicators were 126% [97%; 177%] and 4.66% [3.54%; 7.84%], while only 26.7% of irradiated mice had values below the level characteristic of intact animals. With intraperitoneal administration, the weight of the organ did not reach the physiological norm in 66.7% of animals, amounting to 97 [76; 134] mg (compared with biological control, $p = 0.011$, Mann – Whitney *U* test), respectively, the mass index was in the range of 3.08% [2.65%; 4.12%] ($p = 0.003$).

No pathological disorders were detected in other vital organs (thymus, liver, kidneys, and heart) in irradiated mice, and the mass index values did not differ between the groups. In general, there were no negative effects during cell therapy using allogeneic cells obtained from adipose tissue using both routes of administration.

Thus, the results of the work showed that cell therapy using MMSCs obtained from adipose tissue can be considered as an effective method for treating the bone marrow form of ARS.

To summarize, many unresolved questions remain regarding the conduct of cell therapy for ARS using MMSCs. The subject of discussion is the choice of source for obtaining MMSCs, the optimal time interval for their transplantation after the body irradiation, the route of delivery of the cellular product, the number of administered cells, and the frequency

Table 3. Content of the main forms of leukocytes in the peripheral blood of mice on day 30 after total irradiation at a dose of 7.8 Gy without treatment and with intravenous or intraperitoneal administration of MMSC 24 h after radiation exposure, *Me* [Q_{25}; Q_{75}]
Таблица 3. Содержание основных форм лейкоцитов в периферической крови мышей на 30-е сутки после общего облучения в дозе 7,8 Гр без лечения и с внутривенным или внутрибрюшинным введением ММСК через 24 ч после радиационного воздействия, *Me* [Q_{25}; Q_{75}]

Animal group		Lymphocytes, $\times 10^9$/L	Monocytes, $\times 10^9$/L	Neutrophils, $\times 10^9$/L
Intact		5.2 [4.8; 7.0]	1.3 [1.1; 1.4]	1.8 [1.4; 1.9]
Control 7.8 Gy		1.1 [1.0; 1.6]*	0.6 [0.4; 1.1]	0.7 [0.5; 0.9]**
7.8 Gy + MMSC 30,000 cells/mouse	i.v.	1.68 [1.65; 2.6]*	1.4 [0.5; 1.6]	1.0 [0.5; 1.0]**
	i.p.	1.68 [1.6; 1.7]*	1.1 [0.96; 1.2]	0.8 [0.8; 1.0]**
7.8 Gy + MMSC 60,000 cells/mouse	i.v.	1.2 [0.96; 1.3]*	0.96 [0.8; 1.2]	0.96 [0.96; 1.6]
	i.p.	2.3 [1.0; 2.4]*	1.3 [1.3; 1.8]	1.3 [1.0; 1.4]
7.8 Gy + MMSC. 120,000 cells/mouse	i.v.	1.3 [1.1; 1.6]*	0.8 [0.5; 0.8]	0.78 [0.6; 0.8]**
	i.p.	2.1 [1.8; 2.3]*	1.3 [1.3; 1.4]	1.3 [0.8; 1.4]

Note: * — differences compared to the intact group, $p < 0.01$; ** — $p < 0.05$ (Mann – Whitney *U* test).
Примечание: * — различия по сравнению с интактной группой, $p < 0{,}01$; ** — $p < 0{,}05$ (*U*-критерий Манна – Уитни).

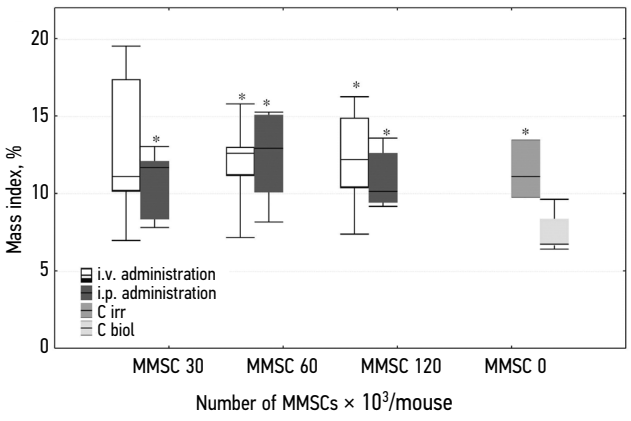


Fig. 6. Lung mass indices in intact mice of the biol group and on day 30 after irradiation at a dose of 7.8 Gy in the Kl group without treatment or with the introduction of MMSCs isolated from mouse adipose tissue (30, 60, and 120 thousand cells/mouse). Differences compared to the *K* biol group: * — $p < 0.05$ (Mann – Whitney *U* test)
Рис. 6. Массовый индекс легких у интактных мышей группы К биол и на 30-е сутки после облучения в дозе 7,8 Гр в группе К обл без лечения или с введением ММСК, выделенных из жировой ткани мыши (30, 60 и 120 тыс. клеток/мышь). Различия по сравнению с группой К биол: * — $p < 0{,}05$ (*U*-критерий Манна – Уитни)

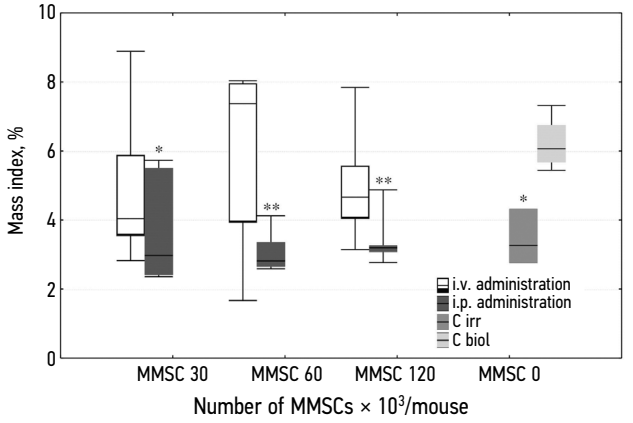


Fig. 7. Mass indices of the spleen in intact mice (K biol) and on day 30 after irradiation at a dose of 7.8 Gy in the Kl group without treatment or with the introduction of MMSCs isolated from mouse adipose tissue (30, 60, and 120 thousand cells/mouse). Differences compared to the biol group: * — $p < 0.01$; ** — $p < 0.05$ (Mann – Whitney *U* test)
Рис. 7. Массовый индекс селезенки у интактных мышей (К биол) и на 30-е сутки после облучения в дозе 7,8 Гр в группе К обл без лечения или с введением ММСК, выделенных из жировой ткани мыши (30, 60 и 120 тыс. клеток/мышь). Различия по сравнению с группой К биол: * — $p < 0{,}01$; ** — $p < 0{,}05$ (*U*-критерий Манна – Уитни)

of administration. Our study utilized a population of cells of adipogenic origin, considering the growing interest in the use of adipose tissue as a source of MMSCs. The obvious advantages are its availability, relatively simple methodologies for isolating and culturing MMSCs, minimal ethical aspects, and a large number of functionally active and viable stem cells [12, 14].

When choosing the timing of cell therapy, we were guided by scientific literature and previous *in vivo* and *in vitro* experiments. Most authors agree that the optimal therapeutic efficiency of MMSCs manifests itself when they are used on day 1 after irradiation, as longer periods reduce the positive effect of cell therapy [15, 16]. Using a mouse model of the bone marrow form of ARS and using cell cultures (mononuclear fraction of bone marrow cells and MMSCs obtained from the subcutaneous adipose tissue of mice), the advantage of MMSCs 24 h after irradiation of mice at an LD of 7.5 Gy over earlier periods of cell therapy for ARS was revealed, as within 4 h after acute radiation exposure, changes occurred in the blood of animals causing toxicity to cellular systems. After 24 h, the cytopathogenic effect of the blood serum of irradiated mice was completely minimized, which was reflected in an increase in the 30-day survival of irradiated animals and positive dynamics in the restoration of bone marrow hematopoiesis and the number of peripheral blood cells when using MMSCs 24 h after irradiation compared with an earlier period of cell therapy (2 h after *X*-ray exposure).

From a clinical standpoint, intravenous delivery of biomedical cell products, including MMSCs, into the body is the most promising and pathogenetically justified method. However, evidence is being gained indicating the accumulation of intravenously administered MMSCs in the lungs and liver, which can result in the formation of microemboli and have serious consequences for the functioning of these organs [17–19]. Therefore, many researchers are searching for alternative methods of administration. Based on literature data proving the ability of intraperitoneally administered MMSCs to migrate into the bone marrow damaged after irradiation, ensuring its reparative restoration [20], we studied the effectiveness of cell therapy for acute radiation injury using both intravenous and intraperitoneal methods of administering the cell product. This outcome revealed that the use of MMSCs of adipogenic origin in both delivery routes contributed to an increase in the life expectancy of mice after lethal irradiation, which is an important indicator of the therapy efficiency, as it enables the reduction of the potential risks of an outcome fatal for the body by adding supportive therapy, particularly antibacterial, anti-inflammatory therapy, etc.

The efficiency of MMSCs after extrasystemic administration is associated with their paracrine effects. MMSCs stimulate the restoration of radiation damage to the bone marrow due to the secretion of hematopoietic factors (granulocyte-macrophage colony-stimulating factor [CSF], macrophage inflammatory proteins 1α and 1β, granulocyte chemotactic factor, keratinocyte chemoattractant, RANTES, interleukin [IL]-17, macrophage CSF, tumor necrosis factor alpha, eotaxin, and interferon-γ-inducible protein 10), leading to a significant increase in the counts of leukocytes in the peripheral blood. Similar data were obtained when using a conditioned medium obtained by culturing MMSCs, which also reduced the severity of ARS and stimulated the restoration of suppressed hematopoiesis, thereby increasing the survival of irradiated mice [20]. The paracrine nature of the MMSCs action is confirmed by studies that have shown the therapeutic efficiency of a cell product based on a population of placental stromal cells (PLX-RAD) when administered intramuscularly and subcutaneously [25, 26]. During the height of ARS, 7.7 Gy irradiated mice treated with PLX-RAD had high levels of nine of 63 human proteins tested, which included major hematopoietic cytokines, such as granulocyte CSF, growth regulation protein GRO, monocyte chemoattractant protein-1 (MCP-1), IL-6, and IL-8, which concentrations reached a maximum on days 6–9 of the experiment. Cytokines mainly associated with leukocyte migration, including MCP-3 (CCL7), epithelial neutrophil attractant (CXCL5), eotaxin (CCL11), and fractalkine (CX3CL1) had similar kinetics [21].

Our data indicates a decrease in the therapeutic effect of MMSCs with an increased number of intravenously administered cells, as assessed by the 30-day survival rate of irradiated mice, which can probably be explained by the “first pass effect through the lungs”. According to I.V. Mayborodin et al. [22], MMSCs accumulated after intravenous administration in the pulmonary capillary network undergo macrophage phagocytosis. The debris of destroyed cells is transported with the blood to other organs. These processes can be much less pronounced with other methods of cellular product delivery.

The results of our study reveal an inverse dependence of animal survival on the dose using the intravenous method of cell product delivery, which can serve as evidence of the need to control the counts of MMSCs administered during radiation damage to the hematopoietic system. Similar data were obtained by K.X. Hu et al. [23], who showed that transplantation of bone marrow-derived MMSCs at a dose of 5×10^7 cells/kg increased the 30-day survival of mice after γ-irradiation at a dose of 8 Gy to 43% with absolute mortality in the control group, and the use of MMSCs in lower

or higher amounts (2.5×10^7 and 1.5×10^8 cells) ensured survival of only 30% and 12% of irradiated mice, respectively. Additionally, M. Bandekar et al. [15] revealed the dependence of the efficiency of cell therapy for ARS in mice on the counts of administered stromal cells derived from Wharton's jelly (WJ-MSC). Intravenous cell transplantation in an amount of 1×10^6 per mouse had the maximum impact on the survival rates of animals after irradiation at a dose of 8.5 Gy, while the protective effect was reduced when applied 0.25, 0.5, or 1.5×10^6 cells. These data are especially important to consider when using drugs that stimulate hematopoiesis in combination with cell therapy.

Thus, the literature data and the results obtained in our study indicate that cell therapy using adipose tissue-derived MMSCs for the bone marrow form of ARS has undoubted prospects. Allogeneic MMSCs, which, unlike hematopoietic stem cells, are characterized by low or no immunogenicity, can provide protection from the death of animals after lethal irradiation, while therapeutic effects appear not only after intravenous transplantation of the cell product but also after intraperitoneal administration. According to the literature, support for hematopoiesis after acute radiation exposure is provided by stimulating the expression of cytokines and chemokines involved in hematopoiesis, which is accompanied by an improvement in hematopoiesis and accelerated restoration of the counts of blood cells in the functional pool and, ultimately, leads to a more rapid recovery of the body after radiation exposure.

CONCLUSIONS

The therapeutic effect of a population of fibroblast-like cells obtained from the stromal-vascular fraction of the subcutaneous adipose tissue of mice and grown in culture for the treatment of the bone marrow form of ARS was studied using a mouse experimental model. The cells were identified as MMSCs because they were adhesive to plastic, forming a confluent monolayer during cultivation, and the process of their osteogenic differentiation *in vitro* was completed by maturation of osteoblasts and the formation of calcium deposits, which indicated their multipotent nature. MMSCs were used for administration to animals at passage levels 3–4.

Cell therapy was performed 24 h after general, relatively uniform *X*-ray irradiation of laboratory mice at a dose of 7.8 Gy. This work was the first to compare the therapeutic efficiency of allogeneic MMSC transplantation using different routes (intravenous and intraperitoneal) of cell suspension administration. A significant increase in the survival rate of mice was revealed during a 30-day follow-up period after irradiation at an LD, which depended on the number of cells administered and the method of delivery of the biomedical cell product. With intravenous administration of MMSCs, the counts of cells in the cell product of 30,000 and 60,000 ensured the survival of 72.7% and 58.3% of irradiated animals, respectively. In the group of untreated mice, 18.2% survived. When the number of cells in the cell product increased to 120,000 cells/mouse, the efficiency of therapy decreased. With the intraperitoneal method of MMSC delivery, the therapeutic effect was registered when MMSCs were administered over the entire dose range, namely, 30,000, 60,000, or 120,000 cells/mouse; cell therapy protected 67%–75% of animals from death.

A decrease in the mortality rate of laboratory mice with acute radiation bone marrow syndrome during allogeneic transplantation of MMSCs was accompanied by an improvement in hematopoietic parameters and accelerated restoration of the counts of blood cells in the functional pool. With the administration of MMSCs on day 30 after irradiation, the values of the main parameters of the hematopoietic system were restored to the initial level in 70%–80% of animals.

Thus, the study results indicate that cell therapy using MMSCs isolated from adipose tissue, with intravenous and intraperitoneal delivery of the cellular product to the irradiated body, protects mice from death after exposure to *X*-ray radiation in LDs, which reduces the radiation damage severity to the hematopoietic system in mice, and has obvious prospects for further development.

ADDITIONAL INFORMATION

Authors' contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

The contribution of each author. E.V. Murzina, N.V. Pak — development of a general concept, research design, data analysis, writing an article; N.V. Aksenova, N.A. Zhirnova — data analysis, preparation of an article for publication; O.M. Veselova, A.A. Khovpachev, N.V. Bely — collection of materials for research, data analysis.

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Вклад авторов. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

Вклад каждого автора. Е.В. Мурзина, Н.В. Пак — разработка общей концепции, дизайн исследования, анализ данных, написание статьи; Н.В. Аксенова, Н.А. Жирнова — анализ данных, подготовка статьи к публикации; О.М. Веселова, А.А. Ховпачев, Н.В. Белый — сбор материалов для исследования, анализ данных.

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